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Amiloride Analogs as ASIC1a Inhibitors

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SUMMARY

Background: ASIC1a, the predominant acid-sensing ion channels (ASICs), is implicated in neurological disorders including stroke, traumatic spinal cord injury, and ALS. Potent ASIC1a inhibitors should have promising therapeutic potential for ASIC1a-related diseases. **Aims:** We examined the inhibitory effects of a number of amiloride analogs on ASIC1a currents, aimed at understanding the structure–activity relationship and identifying potent ASIC1a inhibitors for stroke intervention. **Methods:** Whole-cell patch-clamp techniques and a mouse model of middle cerebral artery occlusion (MCAO)-induced focal ischemia were used. Surflex-Dock was used to dock the analogs into the pocket with default parameters. **Results:** Amiloride and its analogs inhibit ASIC1a currents expressed in Chinese hamster ovary cells with a potency rank order of benzamil > phenamil > 5-(*N*,*N*-dimethyl) amiloride (DMA) > amiloride > 5-(*N*,*N*-hexamethylene)amiloride (HMA) ≥ 5-(*N*-methyl-*N*-isopropyl)amiloride (MIA) > 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). In addition, amiloride and its analogs inhibit ASIC currents in cortical neurons with the same potency rank order. In mice, benzamil and EIPA decreased MCAO-induced infarct volume. Similar to its effect on the ASIC current, benzamil showed a much higher potency than EIPA. **Conclusion:** Addition of a benzyl group to the terminal guanidinyl group resulted in enhanced inhibitory activity on ASIC1a. On the other hand, the bulky groups added to the 5-amino residues slightly decreased the activity. Among the tested amiloride analogs, benzamil is the most potent ASIC1a inhibitor.

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The first two authors contributed equally to this work.

Introduction

Acid-sensing ion channels (ASICs), a member of the degenerin/epithelial sodium channel (DEG/ENaC) superfamily, are proton-gated and voltage-independent cation channels [1,2]. Four genes (ASIC1 to ASIC4) that encode six ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) have been identified [3,4]. ASIC1a and ASIC2a are the predominant ASIC subunits distributed in the central nervous systems (CNS) [2,5,6], while ASIC3 and ASIC1b are predominantly distributed in the peripheral nervous system [7,8].

Tissue acidosis occurs in the nervous system under both physiological and pathological conditions such as synaptic transmission, stroke, and epilepsy. Accumulating evidence has shown that ASICs function as an important acid sensor, and its activation is implicated in several physiological processes including nociception [9–11], mechanosensation [12], and fear behaviors [13–15] as well as in pathological conditions including brain ischemia [16,17] and multiple sclerosis [17,18]. Interestingly, ASIC1a$^{-/-}$ knockout or ASIC1a inhibition, unlike NMDA receptor inhibition, does not cause a significant phenotype change or intolerable side effect [19], suggesting the potential for ASIC1a to serve as a safe and promising target for neurological disorders, especially for stroke [20].

To date, there is a lack of potent and promising small molecule ASIC1a inhibitors that have the therapeutic potential for stroke intervention. Psalmotoxin (PcTx1) is the only specific ASIC1a inhibitor and is an important pharmacological tool for the exploration of ASIC1a function. However, it is not suitable for clinical use for stroke treatment because of its protein nature, which limits

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its effective penetration across the blood brain barrier (BBB). In contrast, small molecule compounds have the advantages over peptides for easy penetration of the BBB and have been used successfully in clinical work for the treatment of various CNS disorders. In this regard, small molecule ASIC1a inhibitors may represent promising therapeutic agents for stroke treatment. Amiloride, used clinically as a diuretic for several decades, is now a commonly used ASIC inhibitor [17]. Importantly, our previous study has shown that amiloride exerted a significant neuroprotective effect in cerebral ischemia [17], suggesting that amiloride may serve as an important lead compound for the development of antistroke drug. Based on amiloride, we performed a structure–activity relationship analysis of several commercially available amiloride analogs as ASIC1a inhibitors, aimed at understanding the structural features needed for future optimization work.

The compounds tested include amiloride, phenamil, benzamil, 5-(*N*,*N*-dimethyl) amiloride (DMA), 5-(*N*-ethyl-*N*-isobutyl)-amiloride (EIPA), 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), and 5-(*N*,*N*-hexamethylene)-amiloride (HMA). We found that benzamil was the most potent ASIC1a inhibitor among the tested amiloride analogs. The current analysis may provide important structure information for the design and development of ASIC inhibitors with improved potency and pharmaceutical properties.

Materials and Methods

Chemicals

The tested compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The catalog numbers are as follows: amiloride (A7410), phenamil (P203), benzamil (B2417), DMA (A4562), EIPA (A3085), HMA (A9561), and MIA (A5585).

Primary Culture of Cortical Neurons

Cortical neurons were cultured from Swiss Webster mice (at embryonic day 16), as described [17,21]. The experimental procedure on using mice for neuronal cell culture was approved by the Institutional Animal Care and Use Committee (Morehouse School of Medicine). Briefly, after anesthesia, the brains were dissected and cortices were incubated in 0.05% trypsin–EDTA for 10 min at 37°C, followed by trituration. The cells were seeded onto poly-l-ornithine-coated culture dishes at a density of 1×10^6 cells per 35 mm dish. Neurons were cultured in MEM supplemented with 10% horse serum and 10% FBS for 24 h, and then maintained in neurobasal medium supplemented with B27 (Invitrogen, San Diego, CA, USA). The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere incubator, and the medium was changed twice per week. Neurons were used for experiments between 10 and 14 days after plating.

Culture of CHO Cells and ASIC1a Transfection

CHO cells were cultured with F-12k medium (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. At ~60% confluence, cells were transfected with rat ASIC1a cDNA fused with a green fluorescence protein (GFP). FuGENE® 6 transfection reagent (Promega, Madison, WI USA) was used to transfect the cells. At 48–72 h after transfection, the GFP positive cells were selected for electrophysiological recordings.

Electrophysiology

As previously described [17,22], whole-cell patch-clamp and fast perfusion techniques were used for ASIC currents recording. The patch pipettes, pulled from borosilicate glass, had a resistance of 2–4 MΩ when filled with the intracellular solution. Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) was used for the whole-cell recording. The data were filtered using Digidata 1320 DAC units (Axon Instruments) at 2 kHz. Data acquisition and analysis were performed using pCLAMP 9.2 software (Axon Instruments). Only the recordings with an access resistance of less than 10 MΩ and a leak current less than 100 pA at −60 mV were included for data analysis [22]. All compounds were co-applied in the pH6.0 solution. A higher concentration was applied after a stable effect of low dose was reached, and at least two overlapped current traces were obtained (which process takes ~3–4 min). The drug washout was performed after a stable effect of highest dose was reached, and at least two overlapped current traces were obtained. 5–10 min washout was allowed until three stable current traces were obtained. The data were normalized to the stable peak current amplitude immediately before the compound was tested.

Focal Ischemia

Middle cerebral artery occlusion (MCAO) was used to induce the transient focal ischemia in mice, as described previously [17]. In brief, male C57BL/6 mice (~25 g, ~10 weeks) were anesthetized using a mixture of 1.5% isoflurane, 70% N_2O, and 28.5% O_2. During surgery, the rectal temperature was monitored and maintained at 37 ± 0.5°C. Transcranical LASER Doppler was used to monitor the change of the cerebral blood flow. Only the mice with a blood flow drop to below 20% of the normal value were used for data analysis. At 24 h after ischemia, the brains were sectioned coronally at 1 mm intervals, and stained with 2% vital dye 2,3,5-triphenyltetrazolium hydrochloride (TTC). Infarct volume was calculated by summing infarction areas (pale) of all sections and multiplying by the thickness of slices. Manipulations and analysis were performed by individual who was blinded to the treatment. Intracerebroventricular injection was performed as described [23] by stereotaxic technique using a 1-μL Hamilton syringe with cannula inserted at 0.5 mm posterior to bregma, 1.0 mm lateral to midline, and 2.5 mm deep relative to the bregma. All manipulations and analysis were performed by individuals blinded to treatment groups.

Solutions and Chemicals

Extracellular solution contained (in mM): 140 NaCl, 5.4 KCl, 20 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$. The osmolarity was adjusted to 320–330 mOsm with sucrose, and the pH was adjusted to 7.4 or 6.0 with NaOH and HCl. Intracellular solution contained (in mM): 140 CsF, 1 $CaCl_2$, 10 HEPES, 11 EGTA, 2 TEA, 4 $MgCl_2$. The osmolarity was adjusted to 290–300 mOsm, and the pH was adjusted to

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7.3 with CsOH and HCl [22]. Amiloride and its analogs were dissolved in DMSO to make 100 mM stock solutions and diluted to 1, 3, 10, 30, and 100 μM in pH 6.0 extracellular solutions for experiments. The final concentration of DMSO in the solutions is 0.1% or less. We tested 0.1% DMSO on each cell and did not observe a significant effect on the ASIC current (data not shown).

Molecular Docking

For all the inhibitors, OMEGA2 [24] was used to generate starting conformations. The amiloride binding pocket is in the extracellular binding domain of ASIC, as shown in the X-ray crystal structure of the co-crystalized protein-amiloride complex [25], was assumed to be the binding pocket for the amiloride analogs. After PROTOMOL was generated with Sybyl-X 2.1 (Tripos International, St. Louis, MO, USA), Surflex-Dock [26] was used to dock all the inhibitors into the pocket with default parameters.

Statistical analysis

All data were expressed as mean ± SE or median as indicated in the figure legends. Sigma plot was used for statistics and dose–response relationship analysis. The dose–response curves were fitted with three parameter logistic nonlinear regression model: $y = a/(1 + (x/x0)^b)$, where a is the relative maximal current, x0 is IC_{50}, and b is the Hill coefficient.

Results

Effects of Amiloride Analogs on ASIC1a Current in CHO Cells

We first examined the effects of amiloride analogs on ASIC1a currents in CHO cells using whole-cell patch-clamp techniques. Cells were clamped at −60 mV, and ASIC1a currents were induced by a pH drop from 7.4 to 6.0. Following the recording of stable ASIC1a currents, various concentrations of amiloride analogs, prepared in pH 6.0 solutions, were tested (Figure 1A,B). As shown in Figure 1A,B, application of amiloride, phenamil, benzamil, DMA, EIPA, MIA, and HMA caused a significant inhibition of ASIC1a currents in a concentration-dependent manner. Data for various concentrations of amiloride and its analogs were then averaged and fitted with the logistic equation to construct the dose–response curves (Figure 1C,D). The rank order of inhibitory potency is as follows: benzamil > phenamil > DMA > amiloride > HMA ≥ MIA > EIPA, with IC_{50} values of 3.50, 6.95, 10.13, 13.50, 17.17, 17.81, and 20.66 μM, respectively (Table 1). These results demonstrate that benzamil is most potent in inhibiting ASIC1a currents among the amiloride analogs tested.

Effects of Amiloride Analogs on ASIC1a Current in Cortical Neurons

ASIC1a is the predominant ASIC subunit in brain neurons. We then examined the effects of amiloride analogs on ASIC currents in cultured mouse cortical neurons. Neurons were clamped at −60 mV, and ASIC1a currents were activated by a pH drop from 7.4 to 6.0. Amiloride analogs, at various concentrations, were tested as indicated in Figure 2A and B, and the dose–response curves were constructed as shown in Figure 2C and D. Similar to ASIC1a currents expressed in CHO cells, ASIC currents in cortical neurons were inhibited by amiloride analogs with the same rank order of potency of benzamil > phenamil > DMA > amiloride > HMA ≥ MIA ≥ EIPA, with IC_{50} values of 2.40, 8.95, 10.68, 13.82, 20.07, 20.76, 20.78 μM, respectively (Table 1). Among all the amiloride analogs, benzamil exhibits the highest potency for the inhibition of ASIC currents in mouse cortical neurons.

Benzamil Protected Brain Against MCAO-Induced Injury

Furthermore, we tested the neuroprotective effect of benzamil and EIPA in a mouse model of transient focal ischemia. Ischemia (60 min) was induced by transient middle cerebral artery occlusion (MCAO). A total of 1 μL artificial CSF (ACSF) alone or 1 μL ACSF-containing benzamil (150 and 500 μM) and EIPA (2 mM) were injected intracerebroventricularly 30 min before the ischemia. The volume for cerebral ventricular and spinal cord fluid for adult mice is estimated to be ~40 μL [27]. Assuming that the infused benzamil was uniformly distributed in the CSF, the estimated concentrations of ~3.5 and 12 μM were expected. The estimated concentration of EIPA was ~48 μM. Infarct volume was determined by TTC staining at 24 h following ischemia (Figure 3A). Ischemia (60 min) produced 59.45% infarct volume in ACSF-injected mice (n = 7), but only 37.46% and 26.41% in benzamil-injected mice (n = 6–8) (Figure 3B). EIPA injection decreases the infarct volume to 29.37% (n = 6) (Figure 3B). We further determined whether benzamil still has protective effect if administered after ischemia. 1 μL ACSF alone or ACSF-containing benzamil (500 μM) were injected intracerebroventricularly 3 h after MCAO. We found that benzamil injection significantly decreased the infarct volume to 33.75% compared with ACSF injection (49%), (n = 5–6) (Figure 3C, D).

Molecular Docking of Amiloride Analogs

To understand the interactions between amiloride analogs and ASIC1a, we performed molecular docking experiments with these inhibitors. The structure of ASIC1a was obtained from recently solved crystal structure of protein–amiloride complex [25]. Firstly, we extract the amiloride which was in the extracellular domain. All the analogs were docked into the original extracellular amiloride binding pocket using Surflex-Dock2.1 program [28] without any bias. The binding pocket was enlarged by 3 Å so that it can accommodate all analogs. This is a common practice in docking studies because of the dynamic nature of protein structures [29]. The various docked structures and poses were evaluated by the program's inner docking score (Total-Score) [30]. The results (Figure 4) show that all inhibitors bind in a similar fashion as amiloride. The first, residue Glu354 plays an important role in generating ionic interactions with the guanidine group of all the inhibitors. Secondly, docking results also suggest that the seemingly repulsive interactions between the large hydrophobic moiety attached to 5-amino group and the positively charged side chain of Lys342 did not have any negative effect on affinity.

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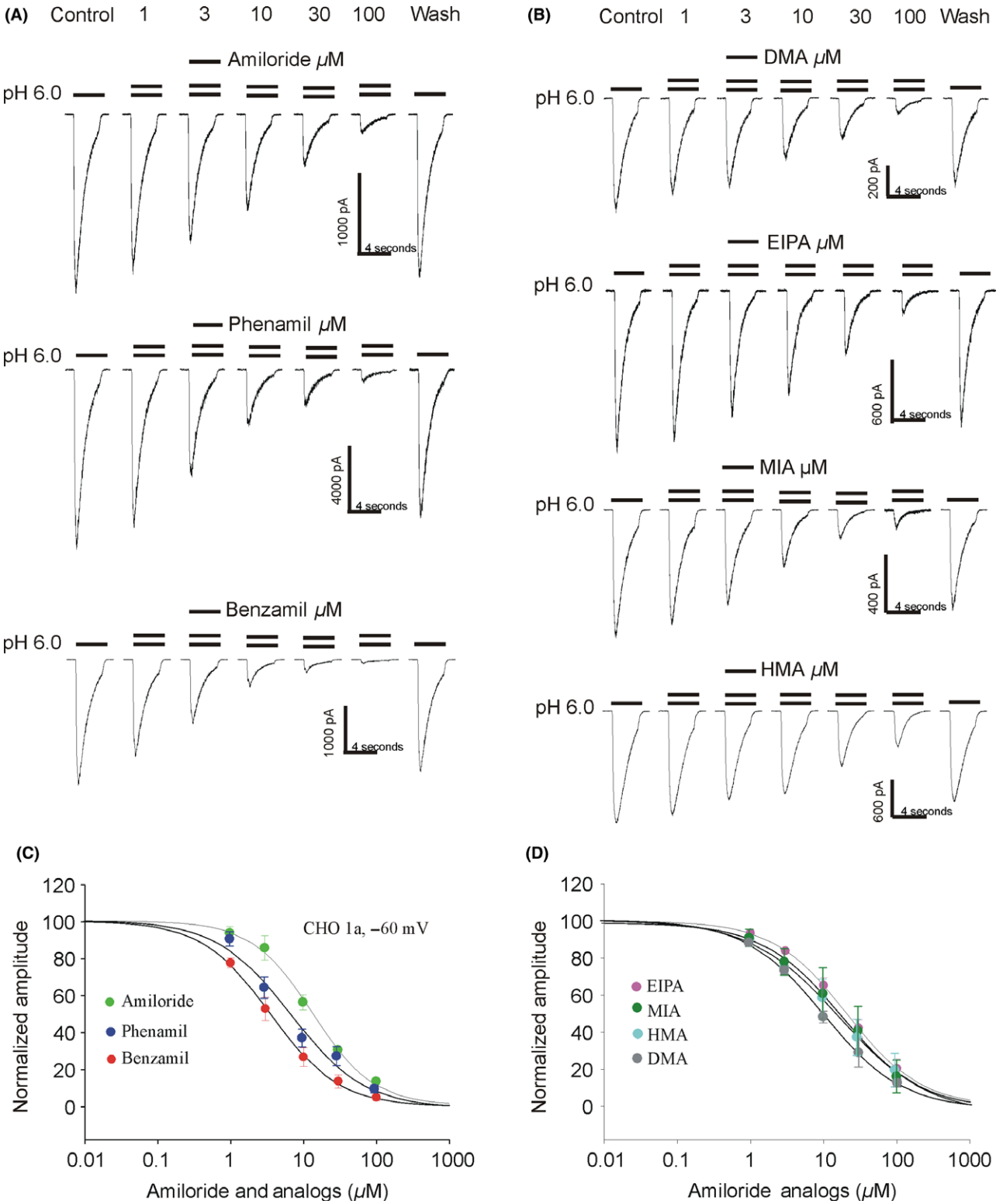

Figure 1 The inhibitory effect of amiloride and its analogs on ASIC1a currents in CHO cells. (**A** and **B**) Representative current traces showing the inhibition of ASIC1a currents by amiloride and its analogs. Amiloride and its analogs were added to a pH 6.0 solution at various concentrations as indicated. (**C** and **D**) Summary data showing concentration-dependent inhibition of the peak amplitude of ASIC1a currents by amiloride and its analogs. Data were expressed as mean ± SE, n = 4–5.

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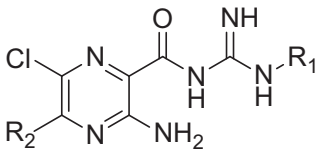
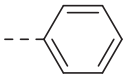
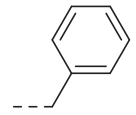
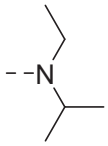
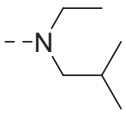
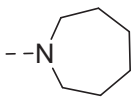
Table 1 Inhibitory effect of amiloride and its analogs on ASIC currents in cortical neurons and CHO cells with stable expression of ASIC1a



	R1	R2	IC50 in cortical neuron	IC50 in CHO cell
Amiloride	H		13.82 ± 1.40 (n = 5)	13.50 ± 1.02 (n = 5)
Phenamil			8.95 ± 1.00 (n = 5)	6.95 ± 1.70 (n = 4)*
Benzamil			2.40 ± 0.12 (n = 5)**	3.50 ± 0.18 (n = 5)**
DMA	H		10.68 ± 1.59 (n = 4)	10.13 ± 0.53 (n = 4)
EIPA	H		20.78 ± 2.72 (n = 4)*	20.66 ± 2.18 (n = 4)**
MIA	H		20.76 ± 1.29 (n = 5)*	17.81 ± 1.75 (n = 5)
HMA	H		20.07 ± 2.86 (n = 4)	17.17 ± 1.33 (n = 4)

IC_{50} is the drug concentration giving half-maximal inhibition of the peak currents. One-way ANOVA analysis of the IC_{50} of amiloride and its analogs. *$P < 0.05$ and **$P < 0.01$ versus amiloride, n = 4–5.

Interestingly, the benzyl group in benzamil seemed to be engaged in cation-pi interactions with Arg191, presumably favoring binding.

Discussion

For several decades, almost all neuroprotective agents, including NMDA receptor antagonists, that showed great promise in preclinical experimental studies failed in clinical trials owing to the limited therapeutic time window and/or intolerable side effects. For example, NMDA receptor antagonists have a limited time window of ~1 h and can cause severe side effects such as schizophrenia. Both factors limit their use in clinical settings [19,31]. Novel and promising therapeutic targets for stroke intervention remain to be identified. Recently, ASIC1a was identified as a promising therapeutic target for stroke treatment [4,17]. During stroke, overstimulation of ASIC1a by brain acidosis causes glutamate-independent neuronal injury in ischemic brain [17]. Knockout of ASIC1a gene or administration of ASIC1a blockers such as amiloride or PcTx1 significantly attenuated acid-induced $[Ca^{2+}]_i$ increase

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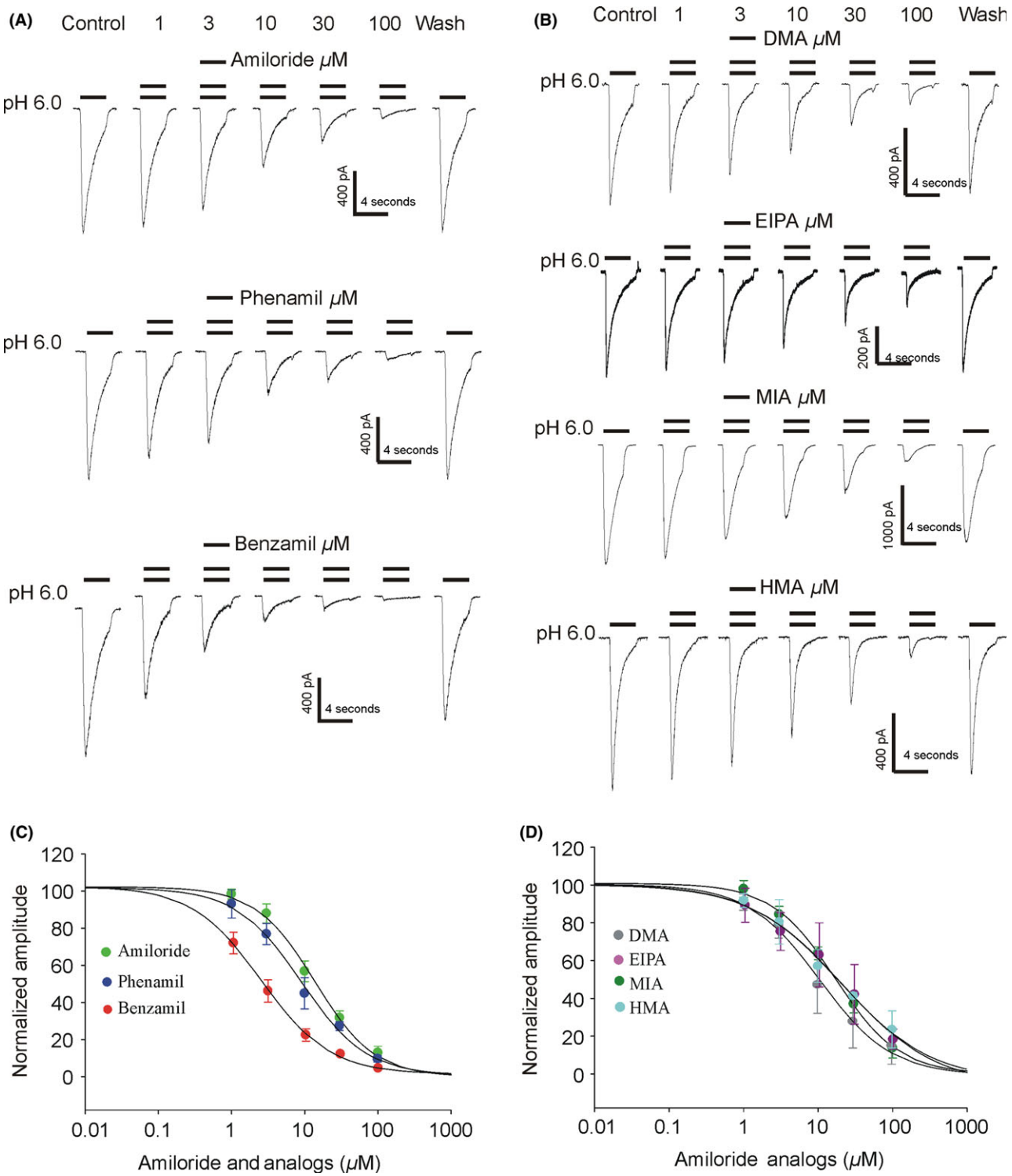

Figure 2 The inhibitory effect of amiloride and its analogs on ASIC currents in cortical neurons. (**A** and **B**) Representative current traces showing the inhibition of ASIC currents by amiloride and its analogs. Amiloride and its analogs were added to a pH 6.0 solution at various concentrations as indicated. The membrane potential was held at −60 mV. (**C** and **D**) Summary data showing concentration-dependent inhibition of the peak amplitude of ASIC currents by amiloride and its analogs. Data were expressed as mean ± SE, n = 4–5.

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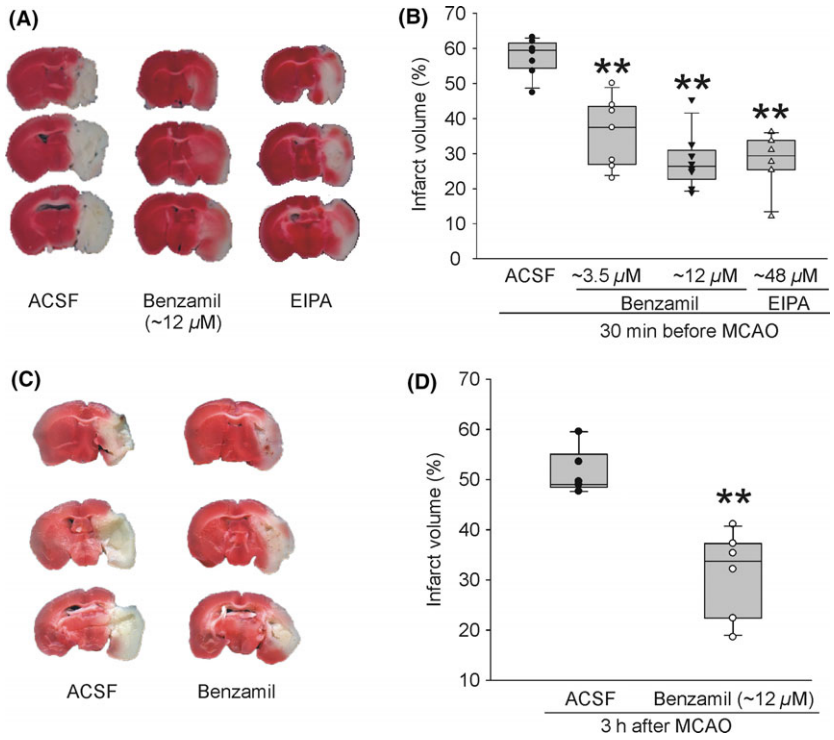

Figure 3 Neuroprotection by benzamil and EIPA in brain ischemia *in vivo*. (**A**) TTC-stained brain sections show infarction area (pale) in brains from artificial cerebrospinal fluid (ACSF)-injected, benzamil (estimated final concentration of ~12 μM)-injected, and EIPA (~48 μM)-injected mice. ACSF, benzamil, and EIPA were applied 30 min prior to middle cerebral artery occlusion (MCAO). (**B**) Reduction in infarction volume in brains from benzamil (~3.5 and 12 μM)- and EIPA (~48 μM)-injected mice. Data are shown as box plots with interquartile, median, and range, n = 6–8 for each group. $^{**}P < 0.01$ compared with ACSF-injected group, One-way ANOVA. (**C**) TTC-stained brain sections show infarction area (pale) from ACSF and ~12 μM benzamil-injected mice. ACSF and benzamil were applied 3 h after MCAO. (**D**) Reduction in infarction volume in brains from benzamil-treated mice. Data are shown as box plots with interquartile, median, and range (n = 5–6). $^{**}P < 0.01$ compared with ACSF-injected group, unpaired *t*-test.

and resultant acidic/ischemic neuronal injury [17]. Importantly, the effective therapeutic time window for ASIC1a inhibition is longer than 5 h in a mouse model of stroke [23], providing a reasonable window of opportunity for stroke intervention. In addition, knockout or inhibition of ASIC1a, unlike NMDA receptors, does not cause significant phenotype changes or side effects [2]. All the evidence suggests that ASIC1a is a promising therapeutic target for stroke treatment.

Small molecule compounds are preferred for the treatment of CNS neurological disorders, owing to their generally good BBB permeability. Currently, there are only few small molecule ASIC1a inhibitors available, including amiloride [17], flurbiprofen/ibuprofen [32], diminazene [33], and local anesthetics [22]. Among these inhibitors, amiloride and diminazene are the most potent ASIC1a inhibitors, with IC_{50} at low micromolar concentrations. Diminazene, an antiinfective drug used in animals, has various side effects including vomiting, diarrhea, and hypotension and can also harm liver, kidneys, and brain. Thus, diminazene's potential use in human is very limited. In contrast, amiloride, a potassium-sparing diuretic, is relatively safe and has been successfully used clinically for management of hypertension and congestive heart failure [34,35]. Thus, we focus on amiloride and structurally related compounds, aimed at identifying more potent ASIC1a inhibitors, as potential therapeutic agents for stroke.

Accumulating evidence has shown that amiloride and some of its analogs have neuroprotective activity. For example, intracerebroventricular injection of amiloride was found to attenuate acidosis-induced cellular damage in CNS neurons through inhibition of ASICs [17,36]. EIPA was found to protect hippocampal neurons and reduce neurological deficits in gerbil global ischemia model [37], although the mechanism remained elusive. Another analog MIA was also shown to protect cells against neonatal brain injury in a mouse hypoxia model [38]. Recently, the therapeutic value of benzamil in treating Huntington's disease (HD) was demonstrated in humans [39] and that the suppression of ASIC1a expression is a proposed mechanism. The above evidence supports the use of amiloride and its analogs as promising neuroprotective drugs. In addition to ASIC1a, benzamil, at low micromolar concentrations, is also an inhibitor of Na^+/H^+ exchanger (NHE), Na^+/Ca^{2+} exchanger (NCX), and epithelial Na^+ channels (ENaCs) [40,41], which effects might also affect the outcome of stroke. The role of NHE in stroke has been well established [4]; for example, gene ablation or pharmacological inhibition of NHE1 has been proved to be protective in animal models of hypoxia/ischemia [38,42,43]. Thus, the inhibition of NHE by benzamil might partly account for the decreased infarct volume observed in the current study. As for the role of NCX, previous studies have demonstrated that knockdown or

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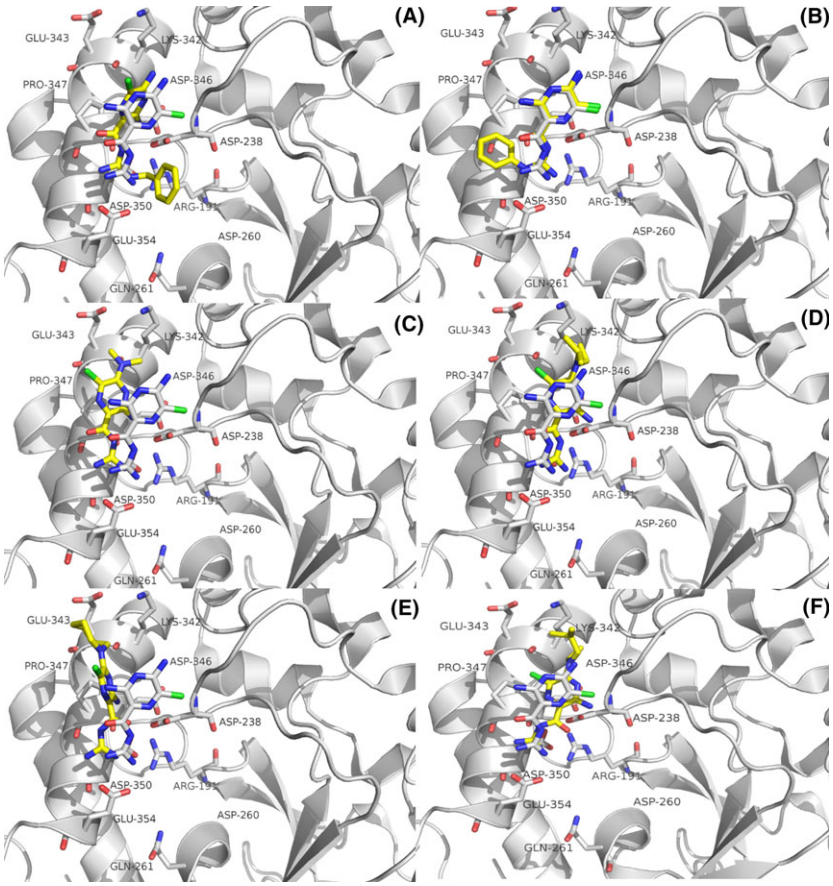

Figure 4 Docking results of ASIC1 inhibitors. White cartoon: ASIC1 protein crystal structure; White sticks: amiloride; Yellow sticks: inhibitors. (**A**) benzamil, (**B**) phenamil (**C**) DMA, (**D**) MIA, (**E**) HMA, (**F**) EIPA.

knockout of the three NCX isoforms including NCX1, NCX2, and NCX3 worsens ischemic brain injury [44–46], suggesting a protective role of NCX in brain ischemia. In this regard, the inhibitory effect of benzamil on NCX may partially counteract its protective effect. However, some pharmacological studies have shown that blockade of NCX results in protective effects. For example, blockade of NCX by SEA0400 and KB-R7943 was reported to be protective in experimental hypoxia/ischemia [47,48]. It is worth mentioning that the potential effects of these compounds on other known or unknown targets may have contributed partially to the effects. For example, in addition to NCX, KB-R7943 inhibits NMDA channels [49], whose blockade is known to result in neuroprotection. ENaCs have been shown to be involved in several important physiological processes including auditory sensation, fluid transport in inner ear, and fertilization and pathological conditions such as hypertension and cystic fibrosis [50]. However, its role in stroke remains elusive. Thus, whether the inhibitory effect of benzamil on NCX affects the stroke outcome remains to be determined.

Addition of a phenyl group to the nitrogen of the guanidinyl side chain of amiloride results in an analog, phenamil, which also has an increased inhibitory activity on ASIC1a currents (~2-fold). Moreover, the introduction of a benzyl group to the nitrogen of the guanidinyl side chain, leading to benzamil, has increased inhibitory efficacy by ~4-fold in comparison with amiloride. DMA is slightly more potent than amiloride; whereas, the introduction of an isobutyl or isopropyl group to amiloride results in slightly less potent analogs MIA and EIPA. The current structure–activity relationship analysis shows that benzamil is the most potent ASIC1a inhibitor among the tested amiloride analogs. Further evaluation of benzamil in MCAO model shows significant efficacy in improving cerebral ischemia outcome. Together, these data suggest that benzamil may represent a promising candidate for ASIC1a-related neurological disorders.

As is true with any docking analysis, future experiments using NMR or site-directed mutagenesis is needed to confirm the putative binding interactions. Along the same line, the synthesis and evaluation of a larger number of analogs will also help confirm the preliminary structure–activity relationship.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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